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14. ABSTRACT

We showed that zinc treatment induced MT expression in LNCaP and C4-2 PCa cells as determined by Western blotting and DNA microarray analysis. Chemotherapy and radiation sensitivity assays of cells after treatment with cisplatin or radiation were performed in the presence, or absence, of 150 microM ZnSO4, and cell viability was measured after 72 hours by MTS viability and clonogenic and flow cytometry assays. The experiments were repeated three times and the data analyzed. We found that increasing concentrations of ZnSO4 upregulated MT expression in a dose-dependent manner. Microarray analysis demonstrated a specific increase in MT expression. Cells treated with zinc demonstrated a significantly decreased sensitivity to cisplatin and radiotherapy compared with controls ($P < 0.05$). Our data have confirmed that treatment of PCa with zinc causes an increase in MT expression, which is significantly associated with resistance to cisplatin chemotherapy and radiotherapy in prostate cancer. Therapeutic targeting of MT may therefore provide a means to overcome resistance to radiotherapy and cisplatin chemotherapy in prostate cancer.

15. SUBJECT TERMS
Advanced prostate cancer, Chemoresistance, Metallothionein 2A, MT 2A Protein kinase C Mu, PKC Mu

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FINAL REPORT

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Title: MT 2A Phosphorylation by PKCMu/PKD Influences Chemosensitivity to Cisplatin in Prostate Cancer.

Introduction

Our preliminary studies had demonstrated that kinase domain of Protein kinase D1, a novel serine threonine kinase, interacts with Metallothionein 2A (MT 2A), which belongs to the family of small molecular weight proteins called metallothioneins (MT) that scavenge trace metals and free radicals and are associated with resistance to chemotherapy and radiation in human cancers (1). Our studies further demonstrated the C4-2 prostate cancer cells that express higher levels of MT compared to its parental LNCaP cells are selectively more resistant to trace metal containing chemotherapy agent cisplatin compared to LNCaP cells (2). Our *in vitro* experiments also demonstrated that MT 2A was phosphorylated by PKD1. Therefore we hypothesized ""*Alteration in MT 2A expression influences chemoresistance to cisplatin in prostate cancer. PKC Mu/PKD kinase activity influences sensitivity to cisplatin by MT 2A phosphorylation in prostate cancer. The expression of MT 2A is quantitatively increased in progressive human prostate cancer"*.

We proposed to establish the stated hypothesis through 3 aims.

Aim 1. To determine that alteration in MT 2A expression influences resistance to cisplatin in prostate cancer.

Aim 2. Inhibition of PKC Mu/PKD kinase activity and its influences on chemoresistance in prostate cancer cells by modulating the phosphorylation of MT 2A.

Aim 3. To quantify and qualitatively evaluate MT 2A protein expression in progressive human prostate cancer.

Body

Task 1: *To determine that alteration in MT 2A expression influences resistance to cisplatin in prostate cancer.*

1a: Effect of MT 2A over expression influences resistance to cisplatin in prostate cancer
We showed that zinc treatment induced MT expression in LNCaP and C4-2 PCa cells as determined by Western blotting and DNA microarray analysis. Chemotherapy and radiation sensitivity assays of cells after treatment with cisplatin or radiation were performed in the presence, or absence, of 150 microM ZnSO4, and cell viability was measured after 72 hours by MTS viability and clonogenic and flow cytometry assays. The experiments were repeated three times and the data analyzed. We found that increasing concentrations of ZnSO4 upregulated MT expression in a dose-dependent manner. Microarray analysis demonstrated a specific increase in MT expression. Cells treated with zinc demonstrated a significantly decreased sensitivity to cisplatin and radiotherapy compared with controls ($P < 0.05$). Our data have confirmed that treatment of PCa with zinc causes an increase in MT expression, which is significantly associated with resistance to cisplatin chemotherapy and radiotherapy in prostate cancer. Therapeutic targeting of MT may therefore provide a means to overcome resistance to radiotherapy and cisplatin chemotherapy in prostate cancer. The details of the results were published in **Urology 67:1341-7, 2006**. Smith DJ, Jaggi M, Zhang W, Galich A, Du C, Sterrett SP, Smith LM, Balaji KC. *Metallothioneins and resistance to cisplatin and radiation in prostate cancer.* (APPENDIX 1)

1b: Down regulation of MT2A by RNAi leads to increased sensitivity to cisplatin.
There are 12 MT isoforms in humans (3). Since the MT members are very similar to each other, it became increasingly difficult to design isoform-specific siRNA and to develop isoform-specific antibody to MT2A. We used the SMARTpool^{TM} siRNA from Dharmacon/Thermo, which consists of four siRNAs targeting MT2A. The antibody was purchased from Dako which recognized all isoforms of human MT proteins. LNCaP and C4-2 cells were transfected the siRNAs and RNAi effect on MT protein was determined by Western blotting. No significant difference was found between targeting and control siRNAs, i.e. the MT protein level did not change significantly after siRNA treatment. We concluded that either the siRNA specificity or the antibody specificity is a continuing problem, which cannot be resolved within the scope of this grant.

Task 2. *Inhibition of PKC Mu/PKD kinase activity and its influences on chemoresistance in prostate cancer cells by modulating the phosphorylation of MT 2A.*

Nuclear expression of MT is associated with resistance to chemotherapy (4) and therefore, we developed an experimental model by promoting nuclear expression of MT as read out assay following alteration of PKD1 activity. To monitor possible interaction between PKD1 and MT family, we chose MT2A which was found interacting with PKD1 in a yeast 2-hybrid test. Two forms of MT2A constructs were made. One is wild-type MT2A tagged by red fluorescence protein (DsRed), which localized to cytoplasm (Fig.1); the other is nuclear MT2A, which fused an SV40 nuclear localization signal (NLS) and tagged by DsRed (Fig.1). The constructs were transfected into LNCaP cells. The stable cells were selected by Fluorescence Activating Cell Sorting (FACS) from pools of at least 10,000 cells, so the population of each cell line is a mixture of various genomic integrations of the MT2A expression vectors.

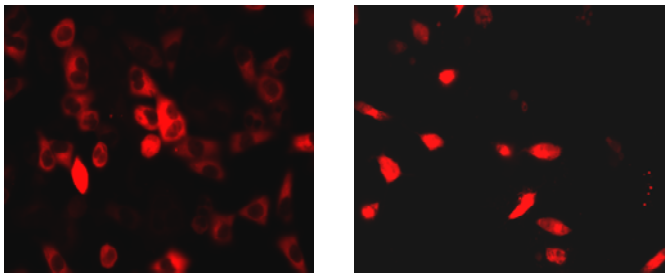

Fig.1. LNCaP stable cell lines that express wild type (Left) and nuclear localized MT2A (Right). The MT2A is tagged by red fluorescence protein. Images were taken by an Olympus IX51 fluorescence microscope at 200X.

The first question we asked was if there was any difference in resistance to cisplatin between cells expressing cytoplasmic or nuclear MT2A. We compared cell viability using MTS assay.

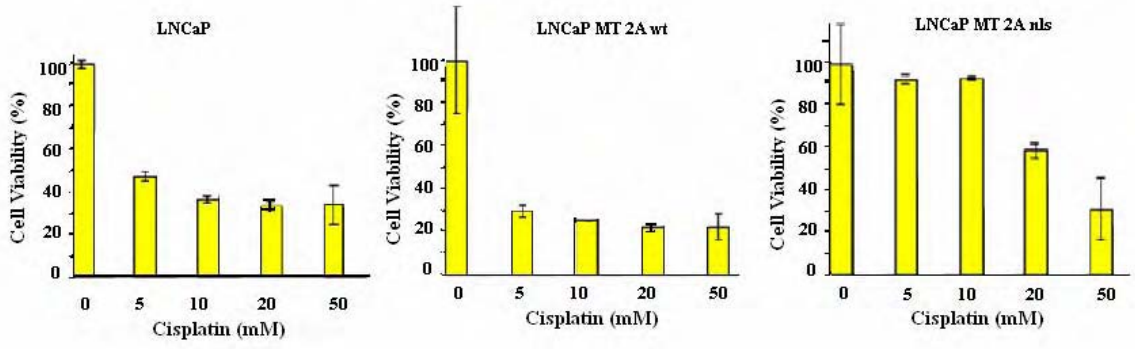

Fig.2. LNCaP cells with nuclear MT2A, but not increased cytoplasmic expression of MT2A, demonstrate increased resistance to cisplatin treatment. Actively growing LNCaP and MT 2A transfected LNCaP cells were plated at 5000 cells per well of a 96 well plate in FBS free RPMI for 24 hours, and media was then changed to RMPI media with 10% FBS and treated cells by adding cisplatin with various concentrations of 0, 1, 5, 10, 20 and 50 mM. Growth and viability assessed by MTS assay at 3 days. Each experiment was carried out in triplicate.

As seen in Fig.2, parental LnCaP cells are sensitive to 5 uM of Cisplatin at which about 50% cells are dead (left panel). Wild type MT2A, which mainly remains in cytoplasm (Fig. 1) did not provide protection against cisplatin (middle panel). In contrast, LNCaP cells nuclear MT2A were not sensitive to 10 uM cisplatin (right panel). Even in the presence of up to 50 uM cisplatin, the survival rate was still higher compared to controls. These results demonstrate that only MT2A in nucleus can protect LNCaP cells from cisplatin. This is consistent with MT's role in protecting DNA damage.

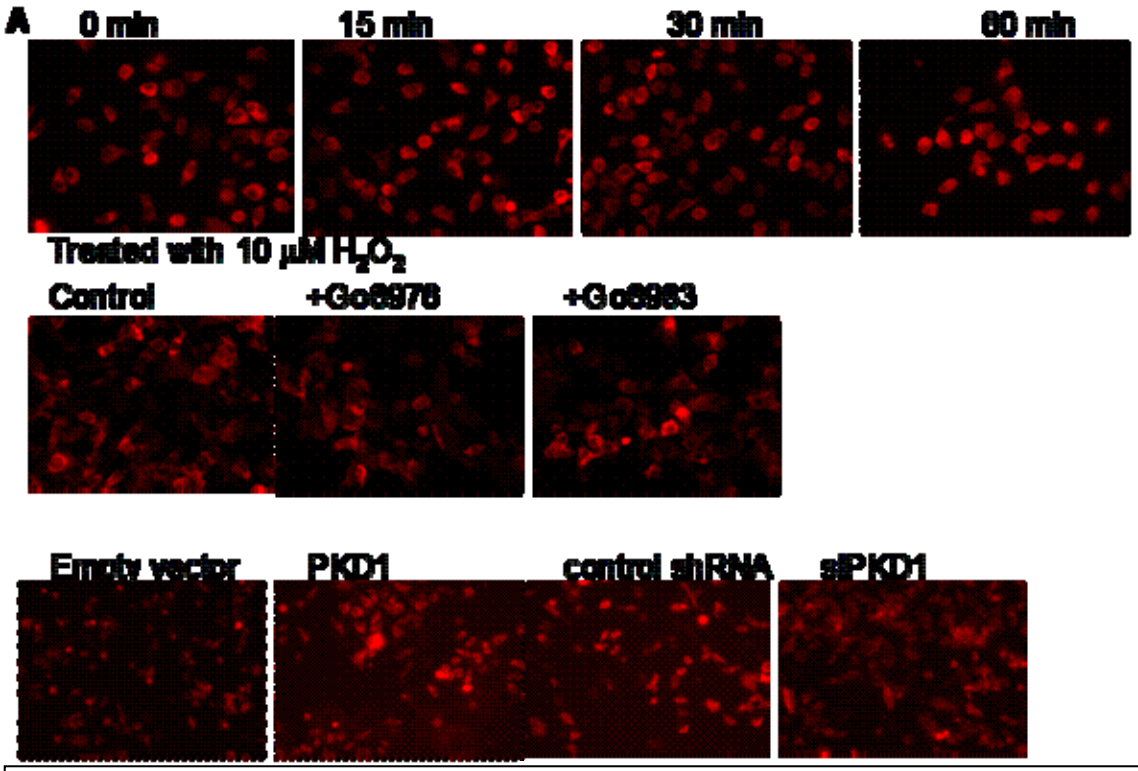

Fig. 3. Manipulation of PKD1 activity did not change wild-type MT2A subcellular localization. (A). Hydrogen peroxide treatment induces MT2A nuclear translocation. MT2A transfected LNCaP cells treated with 10uM H2O2 for indicated times. After 1 hour, MT2A moved into nuclei. This experiment is used as a positive control. (B) Inhibition of PKD1 activity by small molecule inhibitor Go6976 did not induce MT2A translocation. (C) Up- or down-regulation of PKD1 did not affect MT2A subcellular location.

To test if PKD1 activity influences MT nuclear transport, PKD selective inhibitor Go6976 and a control molecule Go6983 (CalBiochem) were added to MT2A transfected LNCaP cells and incubated overnight. However, no significant difference was observed between the two groups (Fig. 3B). The PKD1 levels in wild-type MT2A LNCaP cells were also manipulated by either

over-expression or knockdown by specific siRNA. No significant difference of cytoplasmic or nuclear localization of MT 2A was observed between the treatment groups (Fig.3C).

Next, we measured on LNCaP cell viability and resistance to cisplatin in the presence of PKD selective inhibitor Go6976. Parental and MT2A stable cell lines were treated with a serial concentration of cisplatin in the presence or absence of Go6976. MTS assay were carried out at 3 days. As seen in Fig. 3, in the absence of cisplatin, Go6976 (0.1 uM) shows cytotoxicity to parental (left panel) and wild type MT2A LNCaP (middle panel) cells, either by inhibiting of PKD or by general toxicity. However, LNCaP cells with nuclear MT2A are resistant to the toxicity in the absence of cisplatin (right panel), further suggesting that nuclear MT2A play an important role in detoxification. In the presence of cisplatin, LNCaP cells with nuclear MT2A were sensitive to the even lowest concentration of cisplatin (5 uM), suggesting that inhibition of PKD may reduce cell viability (compare right panels in Figs.2 and 3).

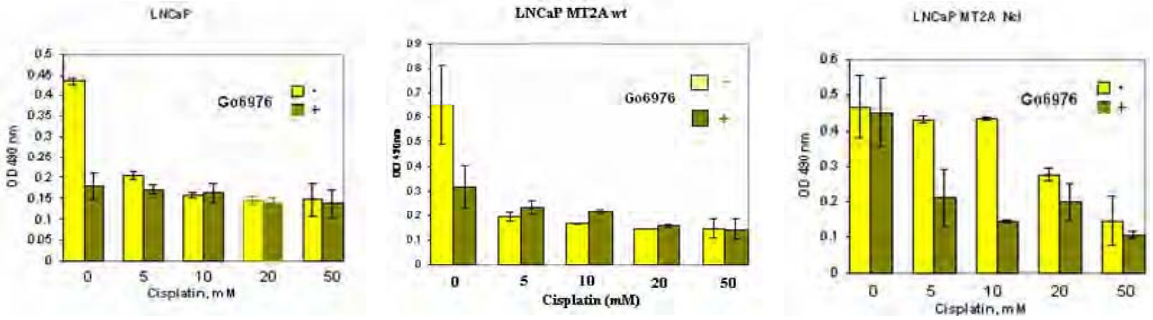

Fig.4. Nuclear expression of MT2A prevents decreased cell viability caused by PKD1 inhibitor GO6976 in LNCaP cells.

It should note that manipulating the expression of PKD1 significantly change cell proliferation, motility and invasion (5). Loss of PKD1 expression was associated with increased expression of the pro-survival molecular markers survivin, beta-catenin, cyclin-D, and c-myc, whereas overexpression of PKD1 resulted in an increase of caspases. However, the inhibitory effect of PKD1 on cell proliferation was rescued by coexpression with beta-catenin, suggesting PKD1 action was mediated by beta-catenin, but not MT2A.

Experiment 2: *Determination of MT 2A phosphorylation using phosphoserine specific antibodies and kinase assays in cells treated with PKC inhibitors*;

We attempted to demonstrate MT2A phosphorylation by PKD1 using GST tagged MT2A, but our study failed to demonstrate MT2A by PKD1 suggesting that PKD1 may influence MT function through subcellular localization.

Task 3: *To quantify and qualitatively evaluate MT 2A protein expression in progressive human prostate cancer.*

Methods: Search patient records, identify cohort of about 20 patients for study, obtain tissue samples, stain and process tissue. QFIA and IHC analysis of slides, analysis of data.
Timeframe: 6-36 months
Deliverable: Demonstrate MT 2A alteration in human prostate cancer tissue.

We have summarized below the results reported in our prior annual reports.

IMMUNOHISTOCHEMISTRY (IHC) ANALYSIS OF MT EXRESSION IN PROSTATE CANCER:
We carried out immunohistochemistry (IHC) studies on a total of 33 human paraffinzed prostate cancer tissue specimens. Adequate grading information was available in 31 of these specimens, of which 20 and 11 specimens consisted of Gleason 5-6 and Gleason 7-10 respectively. MT expression was demonstrable in both nucleus and cytoplasm of prostate cancer cells. However, there was no significant difference in expression of MT between various grades of prostate cancer, pathological stage or serum preoperative prostate specific antigen (PSA) levels (Table 1).

	MT staining		
	absent (1-) (n=10)	present (n=23)	P-value
Gleason 5-6 7-10	 6 (60%) 4 (40%)	 14 (67%) 7 (33%)	1.00
Stage 2 3-4	 8 (80%) 2 (20%)	 19 (83%) 4 (17%)	1.00
PSA 0 - 4 > 4	 - 9 (100%)	 3 (17%) 15 (83%)	0.53

Because we were unable to demonstrate difference in MT expression by IHC studies, we have started to explore MT protein expression by Quantitative Fluorescence Image Analysis (QFIA), which is more sensitive than IHC in detecting differences in protein expression.

QUANTITATIVE FLUORESCENCE IMAGE ANALYSIS OF MT EXRESSION IN PROSTATE CANCER:
QFIA represents a sensitive and reproducible technique for quantifying protein expression. We established the optimum antibody titers and incubation times for analyzing MT expression in LNCaP prostate cancer cells. A concentration of 50,000 LNCaP cells were harvested, fixed and stored at -80^0C. One to three days prior to fluorescence labeling, cyropreserved suspensions were thawed, captured by filtration units, cells blotted and fixed onto slides. Slides are then labeled using a BioGenex autostainer first with the primary antibody (Anti-MT) followed by secondary antibody (goat anti-mouse IgG coupled with Alexa Fluor 488 Molecular Probes). We have determined the optimal primary (1/200, Anti-MT) and secondary antibody (1/100, Molecular Probe) concentrations for detecting metallothionein expression in LNCaP cells. We have also determined optimal antibody incubation times for each (1 hour for each antibody). Negative controls were treated with mouse IgG Isotype control rather than primary antibody. Expression was quantified using the mean pixel intensity of the fluorescence signal following image capture (Fig 5).

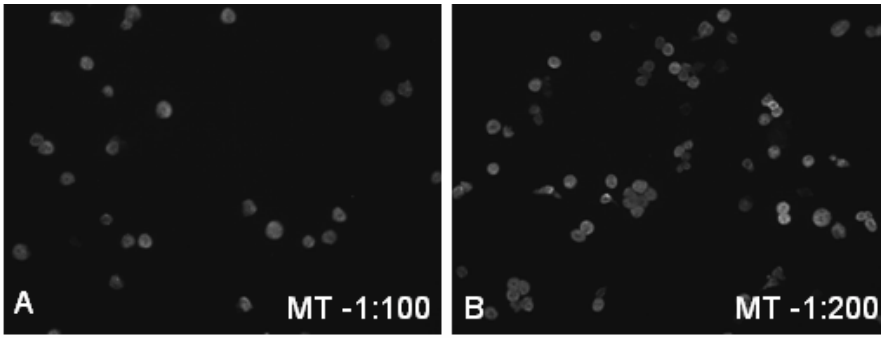

Figure 5; Quantifiable fluorescent signals following labeling of fixed LNCaP cells with MT antibody at concentrations of 1:100 or 1:200. A concentration of 1:200 produces better signal intensity

We attempted to carry out similar optimization studies using parafinized prostate tissues. Unfortunately, we were unable to quantify MT expression using this technique. Therefore, study was limited to IHC analysis.

Key Research Accomplishments

We have confirmed that MT induction by Zn increases resistance to cisplatin in prostate cancer cells. In the process of establishing this result, we have developed an excellent physiologically relevant prostate cancer cell line model system to study the effects of MT expression by Zn. We were unable to demonstrate significant difference in cytoplasmic or nuclear expression of MT between various grades of prostate cancer, suggesting that MT expression may not be associated with prostate cancer progression. Moreover, PKD1 influences MT function through subcellular localization and MT may not be a common substrate for PKD1 kinase activity.

Reportable Outcomes

1. David J Smith, Meena Jaggi, Prema Rao, Anton Galich, Wenguang Zhang and **K.C. Balaji**; Expression of Metallothioneins is Associated with Resistance to Cisplatin and Radiation in Prostate Cancer Cells (Urology. 2006 Jun;67(6):1341-7) (APPENDIX 1)

2. David J. Smith, Meena Jaggi, Lynette M. Smith, **K.C. Balaji**: Expression of Metallothioneins is Associated with Resistance to Both Cisplatin Chemotherapy and Radiation in Prostate Cancer Cells, SCS, AUA, 84th Annual Meeting, Austin, Texas, Abstract #49, 9/05

3. David J Smith, Meena Jaggi, Prema S Rao, Lynette M Smith, **K.C. Balaji,** Expression of Metallothioneins is Associated with Resistance to Both Cisplatin Chemotherapy and Radiation in Prostate Cancer Cells; J Urol, Vol 173, No: 4, Abs 397, 2005

4. Narassa Narayani and K.C. Balaji, Metallothioneins and Prostate Cancer, Chapter 10, in Metallothioneins in Biochemistry and Pathology, Edited by Poalo Zatta, Published by World Scientific, 2008 (APPENDIX 2)

Conclusions

MT expression is strongly associated with resistance to cisplatin chemotherapy in human prostate cancer cells.

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2) Cheng Du, PhD, Research Assistant Professor, Division of Urology, Department of Surgery, University of Massachusetts Medical School, Worcester, MA

ELSEVIER

Appendix 1

METALLOTHIONEINS AND RESISTANCE TO CISPLATIN AND RADIATION IN PROSTATE CANCER

DAVID J. SMITH, MEENA JAGGI, WENGUANG ZHANG, ANTON GALICH, CHENG DU, SAMUEL P. STERRETT, LYNETTE M. SMITH, AND K. C. BALAJI

ABSTRACT

Objectives. The metallothioneins (MTs) are a family of small molecular weight trace metal and free radical scavenging proteins well established to play a role in the resistance to chemotherapy and radiotherapy in human cancers. MT gene expression is upregulated in response to the presence of metal ions such as zinc. Because prostatic tissue has the greatest concentration of zinc in the human body, in this study we analyzed the effect of MT induction by zinc in prostate cancer (PCa).

Methods. The activation of MT gene expression in response to zinc treatment in LNCaP and C4-2 PCa cells was shown by Western blotting and DNA microarray analysis. Chemotherapy and radiation sensitivity assays of cells after treatment with cisplatin or radiation were performed in the presence, or absence, of 150 μM $ZnSO_4$, and cell viability was measured after 72 hours by MTS viability and clonogenic and flow cytometry assays. The experiments were repeated three times and the data analyzed.

Results. Increasing concentrations of $ZnSO_4$ upregulated MT expression in a dose-dependent manner. Microarray analysis demonstrated a specific increase in MT expression. Cells treated with zinc demonstrated a significantly decreased sensitivity to cisplatin and radiotherapy compared with controls ($P < 0.05$).

Conclusions. Our data have confirmed that treatment of PCa with zinc causes an increase in MT expression, which is significantly associated with resistance to cisplatin chemotherapy and radiotherapy in PCa. Therapeutic targeting of MT may therefore provide a means to overcome resistance to radiotherapy and cisplatin chemotherapy in PCa. UROLOGY **67:** 1341–1347, 2006. © 2006 Elsevier Inc.

The prostate gland is distinct from other organs in the human body by its unusually high concentration of zinc (150 μg/g wet weight compared with 20 to 50 μg/g wet weight in other organs).[1] Among the genes responsive to zinc induction, metallothioneins (MTs) are an important family of proteins associated with resistance to chemotherapy and radiotherapy in human cancers.[2] Several reports have demonstrated MT overexpression to be a useful prognostic factor for tumor progression and implicated in causing resistance to chemotherapy in a variety of human cancers.[3–5] The MT 1A, MT 1E, MT 1X, and MT 2A isoforms of the MT gene have been found in normal human prostatic tissue.[6] Because MTs are trace metal-responsive genes, we evaluated the effect of MT induction by zinc on resistance to radiotherapy and cisplatin treatment in prostate cancer (PCa) cells, which may provide unique opportunities to manipulate the cellular events in a prostate cell.

This work was supported by a seed grant from the University of Nebraska Foundation, Omaha, Nebraska and by a grant from the Department of Defense/PC Research grant PC040484.

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MATERIAL AND METHODS

Western Blotting of $ZnSO_4$ Treated Cells

LNCaP and C4-2 PCa cells were plated in six-well plates (3×10^5 cells/well) in the presence of 0, 25, 50, 100, 150 μM $ZnSO_4$ or 150 μM $MgSO_4$ and grown for 72 hours. The cells were then lysed, and Western blotting was performed as previously described[7] using E9 MT antibody (Dako, Carpinteria, Calif), anti beta-actin antibody (Sigma Chemical, St. Louis, Mo), and horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, Wis).

Oligonucleotide Array Gene Expression Analysis

DNA microarray experiments were done using 40 μg total RNA extracted from LNCaP or C4-2 cells treated with or with-

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doi:10.1016/j.urology.2005.12.032

out 150 μM $ZnSO_4$. DNA oligonucleotide microarray slides were obtained from our institutional core facility. These slides contain DNA oligomers representing 12,144 known human genes and a selection of expressed sequence tags of unknown genes. Hybridizations were performed by the microarray core facility according to standard protocols and scanned with a ScanArray 4000 confocal laser system (Perkin-Elmer, Wellesley, Mass). The background fluorescence was subtracted and normalization and filtering of the data were performed using the QuantArray software package (Perkin-Elmer). Next, expression ratios were calculated for each feature and linear regression values calculated for the data in each experiment.

Cell Growth and Viability Assay

The effect on cell growth and viability of C4-2 PCa and CP70 ovarian cancer cells treated with increasing concentrations of $ZnSO_4$ or $MgSO_4$ (0 to 200 μM) were studied by MTS assay using the manufacturer's protocol (Promega).

Chemotherapy and Radiotherapy Sensitivity Assays

LNCaP and C4-2 cells were plated in 96-well plates (5×10^3 cells/well) in the presence or absence of 150 μM $ZnSO_4$. The cells were then incubated at 37°C for 24 hours, before the addition of cisplatin at concentrations of 0, 2, 5, 10, 20, and 50 μM and incubated for an additional 72 hours. Cell growth and viability was measured by the MTS assay, absorbance readings were measured at wavelengths of 490 and 655 nm, and these values were then normalized to the absorbance of untreated control cells. The experiments were performed in triplicate and the mean values compared.

For the radiation experiments, we used a dose of 5 Gy of gamma-radiation (Gammaradiator 100), which caused significant cell mortality (30% to 40% of treated cells) but not complete cell death, and cell growth and viability were measured by MTS assay after 72 hours, as described above. The experiments were performed in triplicate, and the mean values of the irradiated and nonirradiated cells treated with or without $ZnSO_4$ were compared after correction of zinc-induced mild cytotoxicity.

Cell Cycle and Apoptosis Assay by Flow Cytometry

The LNCaP and C4-2 cells were grown at a density of about 5×10^5 cells on 25-cm^2 dishes, as described previously, and treated with 10 μM cisplatin or a gamma-radiation dose of 5 Gy, in the presence or absence of 150 μM $ZnSO_4$ for 48 hours. The cells were trypsinized, centrifuged, washed in phosphate-buffered saline, fixed with cold ethanol for 15 minutes, resuspended, and stained with Telford reagent at 4°C overnight.[8] All flow cytometry measurements were done using the FACS Calibur flow cytometer (BDIS, San Jose, Calif), and data from at least 10,000 cells were collected using ModFit LT, version 2.0, for cell cycle and apoptosis. Apoptosis was reported as a percentage of cells in the sub-G_0 peak.

Clonogenic Assays

LNCaP and C4-2 cells were plated in six well plates (3×10^5 cells/well) and treated with 10 μM cisplatin or a gamma-radiation dose of 5 Gy (Gammaradiator 100) in presence or absence of 150 μM $ZnSO_4$ for 48 hours. Drug-containing media were removed, and the cells were incubated in drug-free media for 2 weeks. The colonies were then fixed, stained by Protocal stain (Fisher Scientific, Middletown, Va) according to the manufacturer's protocol, and counted.

Statistical Analysis

A mixed analysis of variance model was used to compare the mean values between groups. Chi-square tests were used to compare the amount of apoptosis between groups. When significance was found between groups, pairwise tests were conducted, and the Bonferroni method was used to adjust the *P* values for multiple comparisons. The Wilcoxon rank sum test was used to compare the clonogenic assay results between the cisplatin and cisplatin plus zinc groups. $P < 0.05$ was considered statistically significant. Statistical Analysis System (SAS Institute, Cary, NC) software was used for the analysis.

RESULTS

Effect of Zinc on Cell Growth and Viability of PCa Cells

As shown in Figure 1A, although the concentration of $ZnSO_4$ up to 200 μM did not significantly affect the viability of the PCa cells, most control ovarian cancer CP70 cells died at concentrations greater than 50 μM. These results demonstrate the characteristic tolerance of C4-2 PCa cells to high concentrations of zinc.

Induction of MT Expression by $ZnSO_4$

Treatment of LNCaP and C4-2 cells with increasing concentrations of $ZnSO_4$ induced MT protein expression (Fig. 1B). The expression of protein kinase D1, used as a control, was unaltered by $ZnSO_4$. The addition of 150 μM $MgSO_4$ to LNCaP and C4-2 cells did not affect the expression of MT, confirming the specificity of induction of MT by zinc (Fig. 1C).

Microarray Analysis Confirmed MT Induction in LNCaP and C4-2 Cells by $ZnSO_4$

The gene induction by $ZnSO_4$ in both LNCaP and C4-2 cells showed similar patterns (Fig. 1D,E). Linear regression values were greater than 0.9 for all data sets, indicating a high degree of linearity. Only 40 (0.33%) and 26 (0.21%) of 12,144 genes were differentially expressed between the treated and untreated samples in the LNCaP and C4-2 cells, respectively. Within the tiny fraction of the differentially expressed genes between the treated and untreated samples, MT 1 and MT 2 were strongly upregulated in both cell lines. The MT 3 fluorescence signal was low in both cell lines suggesting low cellular expression, which concurs with the published data that MT3 is expressed predominantly in neuron cells.[9] Although a number of genes were upregulated, only two genes in LNCaP and nine genes in C4-2 were significantly downregulated, none of which were highly expressed (Tables I and II). We have established that treatment of PCa with zinc is an excellent in vitro model to study the effects of MT induction.

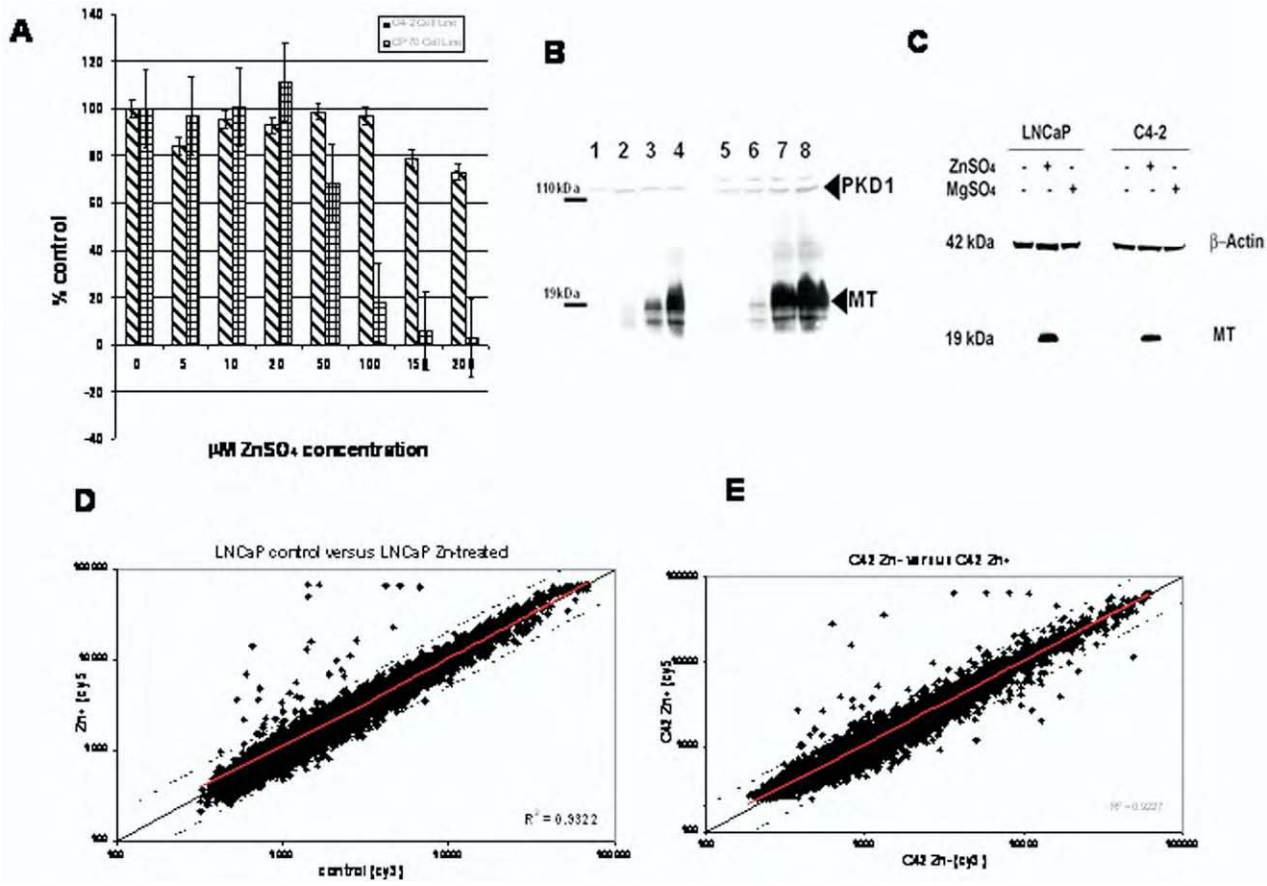

FIGURE 1. *(A) C4-2 PCa cells characteristically tolerated treatment with high concentrations of $ZnSO_4$. C4-2 PCa cells and CP70 ovarian cancer cells (control) were grown to 80% in presence of $ZnSO_4$. Cells were then incubated at 37°C for 72 hours. Cell growth and viability were measured by MTS assay using the aqueous nonradioactive cell proliferation assay kit (Promega). Although C4-2 PCa cells did not demonstrate significant decreases in viability despite high concentrations of $ZnSO_4$ (up to 200 μM), most CP70 ovarian cancer cells died at concentrations greater than 50 μM. (B,C) MT expression was upregulated by $ZnSO_4$, but not $MgSO_4$. LNCaP and C4-2 cells were plated in six-well plates 24 hours before the addition of increasing concentrations of $ZnSO_4$ or $MgSO_4$. After 72 hours of incubation, cells were lysed and analyzed by Western blotting (as described in the Material and Methods section). (B) Lanes 1 to 4 show LNCaP cells and lanes 5 to 8 show C4-2 cells treated with 0, 50, 100, and 150 μM $ZnSO_4$. Positions of protein markers shown on left. Protein kinase D1 was visible as band 120 kDa in size in all lanes, and its expression was not affected by zinc treatment. MT expression clearly increased in a dose-responsive manner. Induction of MT expression in response to increasing $ZnSO_4$ concentration shown by increase in band size at around 10 kDa. (C) Induction of MT expression in LNCaP and C4-2 cells treated with $ZnSO_4$, with no induction of MT in $MgSO_4$ treated cells. (D,E) Alterations in gene expression in LNCaP and C4-2 cells by 150 μM $ZnSO_4$. Scatter plots show gene expression ratio changes in (D) LNCaP and (E) C4-2 cell lines in presence or absence of 150 μM $ZnSO_4$. Dots above upper dashed line indicate genes expressed significantly (more than 2 logs) more in cells treated with zinc. MT isoforms visible as most highly upregulated genes at top of each plot. Dots within dashed lines represent genes whose expression did not change significantly (less than 2 logs). Dots below lower dashed line indicate genes expressed significantly (more than 2 logs) less in cells treated with zinc. R^2 values indicate linear regression of data points.*

Treatment of PCa Cells with Zinc Induces Cisplatin Resistance

We incubated the LNCaP and C4-2 cells for 72 hours in the presence or absence of 150 μM $ZnSO_4$ and increasing concentrations of cisplatin. The inhibitory concentration (IC_{50}) for cisplatin-induced cell death was 10 μM in cells not treated with $ZnSO_4$ and was therefore selected as the study concentration for the clonogenic and apoptosis assays (Fig. 2A,B). The cells treated with $ZnSO_4$ were significantly ($P < 0.005$) more resistant to cisplatin at all study concentrations, strongly suggesting an association between MT induction and resistance to cisplatin in PCa.

Zinc Increased PCa Cell Resistance to Gamma Radiation

In addition to cisplatin resistance, MTs contribute to radiation resistance by scavenging the free radicals generated by ionizing radiation.[10] We ex-

TABLE I. ***Balanced expression ratios of gene expression from microarray analysis of LNCaP cells treated with 150 μM $ZnSO_4$****

Gene Name	Genbank ID	Balanced Expression Ratio
Upregulated		
Hemopexin	J03048	26.1
Metallothionein 1L	S68954	21.0
Fetal brain cDNA H62G	Z70717	15.9
Metallothionein 2A	NM 005953	12.9
Metallothionein (all)	X64177	9.8
Putative glycine-*N*-acyltransferase (GAT)	NM 005838	7.9
Metallothionein 1G	S68954	6.0
Melanoma antigen, family B4 (MAGEB4)	NM 002367	5.9
Metallothionein 1R	X97261	5.1
Deleted in colon cancer (DCC)	S81335	5.0
Downregulated		
Stanniocalcin 2	NM 003714	3.4
FLJ22209 fis, clone HRC01496	AK025862	2.0

** All genes with >5 logs upregulation and >2 logs downregulation in response to 150 μM $ZnSO_4$ treatment shown.*

TABLE II. ***Balanced expression ratios of gene expression from microarray analysis of C4-2 cells treated with 150 μM $ZnSO_4$****

Gene Name	Genbank ID	Balanced Expression Ratio
Upregulated		
Metallothionein (all)	X64177	46.1
Hemopexin	J03048	33.4
Fetal brain cDNA H62G	Z70717	31.6
Metallothionein 1L	S68954	24.3
Metallothionein 1R	X97261	15.5/4.8
Metallothionein 1G	S68954	11.9
Putative glycine-*N*-acyltransferase (GAT)	NM 005838	11.4
Melanoma antigen, family B 4 (MAGEB4)	NM 002367	10.6
Deleted in colon cancer (DCC)	S81335	9.3
Metallothionein 2A	NM 005953	9.3
KIAA0939 protein	AB023156	6.7
Solute carrier family 16 member 2SLC16A2	NM 006517	6.5
Member RAS oncogene family RAB33A	NM 004794	5.8
cDNA DKFZp434C107	AL133645	5.0
Downregulated		
G protein alpha 13 (GNA13)	NM 006572	2.4
Small GTP-binding protein (RAB1B)	U66621	2.4
Acid phosphatase 1 soluble (ACP1), transcript variant A	NM 004300	2.3
BAI1-associated protein 2 (BAIAP2), transcript variant 1	NM 017450	2.2
Membrane metallo-endopeptidase (MME), transcript variant 2b	NM 007289	2.1
Hypothetical protein FLJ20523	NM 017862	2.1
Oxoglutarate dehydrogenase (lipoamide) (OGDH)	NM 002541	2.1
Myc-associated zinc-finger protein of human islet	D85131	2.0
XS137	Z36875	2.0

** All genes with >5 logs upregulation and >2 logs downregulation in response to 150 μM $ZnSO_4$ treatment shown.*

plored whether induction of MT expression increased the resistance of PCa cells to gamma radiation (Fig. 2C). The difference in the resistance to radiation between zinc-treated and untreated cells was highly significant ($P < 0.001$), indicating that the addition of 150 μM zinc induced a protective effect against radiation damage in both cell lines.

Treatment of PCa Cells with Zinc Decreased Cisplatin and Radiation-Induced Apoptosis

Flow cytometric analyses were performed, and the apoptotic cell populations in the sub-G_1 phase were quantified. A significant decrease in cell death occurred in LNCaP and C4-2 cells treated with cisplatin (LNCaP, 10%; LNCaP plus cisplatin,

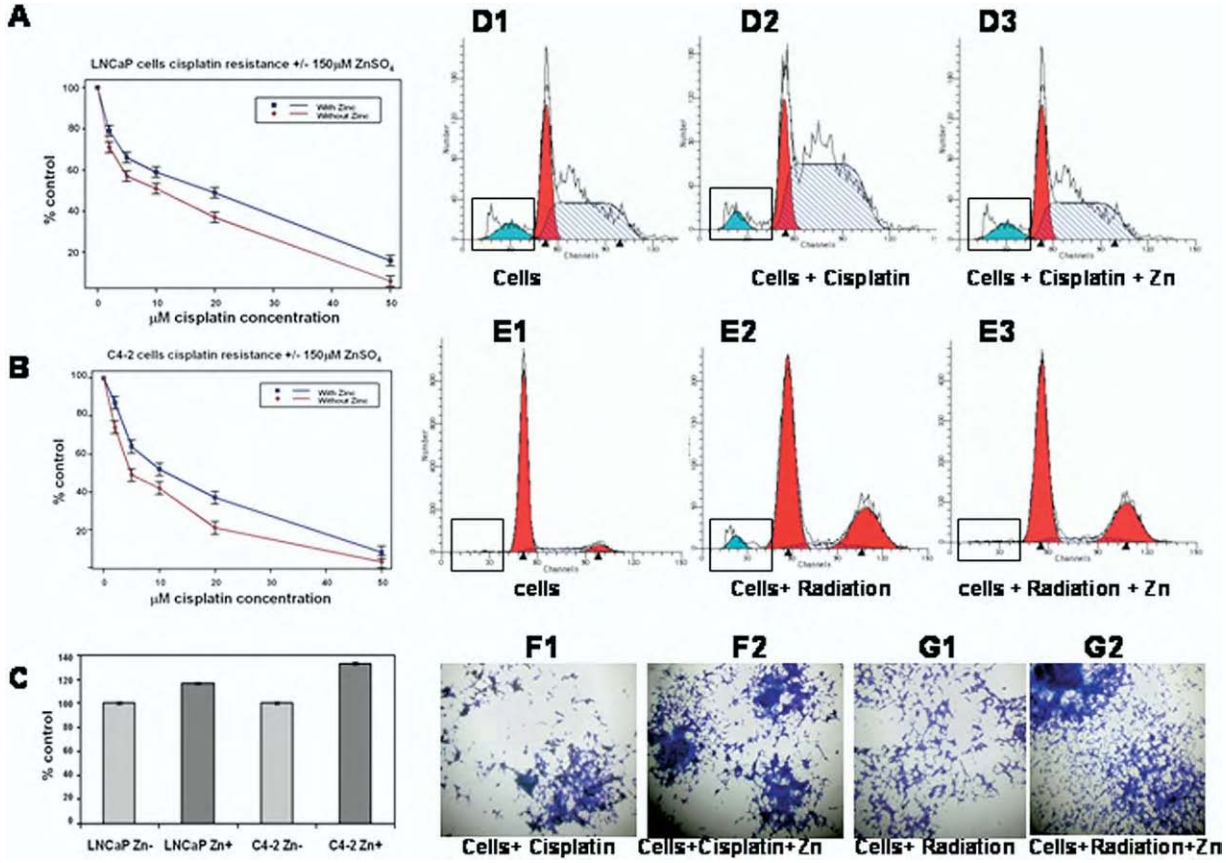

FIGURE 2. *Cisplatin dose-response experiments on (A) LNCaP and (B) C4-2 cells. Experiments performed at cisplatin doses of 0 to 50 μM, with cell viability measured at 3 days. Each experiment was performed in triplicate. Cell growth and viability were measured by MTS assay, absorbance readings were taken at wavelengths of 490 and 655 nm, and average absorbance for each cell line and drug concentration was calculated. Error bars indicate mean standard error. Data points for zinc-treated cells shown as squares and those for untreated cells as diamonds. (C) Radiation sensitivity of LNCaP and C4-2 cells in presence of 150 μM $ZnSO_4$. LNCaP and C4-2 cells were grown to 80% confluence, plated in 96-well plates, and incubated overnight in the presence or absence of 150 μM $ZnSO_4$, as described in previous experiment. Cells were then exposed to 5 Gy of gamma-radiation, grown for an additional 72 hours, and cell growth and viability were measured by MTS assay. Experiments were performed in triplicate, and mean values compared. Results shown as bar graph comparing cells exposed to radiation in presence or absence of 150 μM $ZnSO_4$. Error bars show standard error. (D,E) Analysis of apoptosis by flow cytometry. C4-2 cells treated with 10 μM cisplatin or 5-Gy radiation in presence or absence of 150 μM of $ZnSO_4$ for 48 hours were analyzed. Significant decrease in apoptosis (Box, sub-G_O peak) in cells treated with $ZnSO_4$ compared with untreated cells (C4-2 cells, only 8.73%; C4-2 cells plus cisplatin, 14.24%; C4-2 cells plus cisplatin plus zinc, 9.12%; P <0.001 [D1 to D3], C4-2 cells, 0.34%; C4-2 cells plus radiation, 6.35%; C4-2 cells plus radiation plus zinc, 1.13%; P <0.001 [E1 to E3]). (F,G) Representative view of clonogenic assay demonstrating increased number of colonies in C4-2 cells treated with 10 μM cisplatin (F1 to F2) or 5-Gy radiation (G1 to G2) and 150 μM of $ZnSO_4$ compared with cells not treated with $ZnSO_4$.*

30.64%; LNCaP plus cisplatin plus zinc, 16.64%; C4-2, 8.73%; C4-2 plus cisplatin, 14.24%; C4-2 plus cisplatin plus zinc, 9.12%; $P < 0.001$; Fig. 2D1 to 2D3) or radiation (LNCaP, 1.05%; LNCaP plus radiation, 9.26%; LNCaP plus radiation plus zinc, 1.17%; C4-2, 0.34%; C4-2 plus radiation, 6.35%; C4-2 plus radiation plus zinc, 1.13%; $P < 0.001$; Fig. 2E1 to 2E3) after pretreatment with zinc compared with untreated cells. No changes were found in the cell cycle phases. In an effort to establish that inhibition of apoptosis by $ZnSO_4$ in PCa cells treated with cisplatin or radiation persists to influence cell growth, we performed clonogenic assays. The number of colonies was significantly increased in LNCaP and C4-2 cells treated with zinc and cisplatin (median 104.5, range 102 to 108, median 38.5, range 30 to 45; $P = 0.03$, Fig. 2F1 to 2F2) and increased twofold in the zinc and radiation group compared with the cisplatin or radiation-treated cells without zinc (LNCaP cells, 99 and 56; C4-2 cells, 98 and 48; Fig. 2G1 to 2G2).

COMMENT

The results of the present study have established an excellent in vitro cell line model to study the effect of MT expression in PCa. The results from our microarray analysis have confirmed that zinc

treatment induces 0.33% of the 12,144 genes studied, of which the predominant are the MTs. Others have studied the effect of MT induction using zinc chloride on the resistance to cisplatin in mice.[11] Although such studies have helped to establish the role of MTs in the resistance to chemotherapy, the use of compounds such as mercury at study concentrations are usually toxic to humans and may not be translatable to human use. Because our experimental system used a high zinc concentration similar to that of the human prostate, the results may be more readily translated to clinical settings. Because PCa is a multifocal disease, high concentrations of zinc in normal-appearing prostatic tissue surrounding the cancerous tissue may influence the phenotypic behavior of the neighboring cancerous tissue. Although our experiments simulated the in vivo conditions of a high zinc concentration, the caveat is that zinc concentrations within the heterogenous population of cells in the prostate vary; therefore, our in vitro experiments may not have truly replicated the prostate microenvironment.

The results of our study have demonstrated a significant association between MT induction and resistance to cisplatin in PCa cells. Others have demonstrated the presence of an MT-like zinc-binding protein in PCa cell lines exhibiting relative resistance to cisplatin, and nuclear localization of MT has been shown to be associated with cisplatin resistance in PCa cell lines.[12,13] Although additional work is necessary to understand the mechanistic basis of MT-induced resistance to cisplatin chemotherapy, it is conceivable that MTs may function as an effective scavenger of trace metals, including platinum, which may render cisplatin ineffective in PCa. Although no specific MT inhibitor has yet been described, inhibition of cysteine synthesis by propargylglycine has been shown to significantly reduce MT induction in mice inoculated with human or murine bladder tumor cells, which markedly enhanced the antitumor activity of cisplatin and other drugs.[14] Because MTs may be therapeutically manipulated, additional studies are needed to explore MTs as a biomarker for the chemosensitivity of PCa to cisplatin treatment and possibly improve the efficacy of cisplatin as a radiosensitizer.

Our results have also demonstrated that MT induction is associated with significant resistance to radiotherapy in PCa. Radiotherapy fails in a few patients with PCa. Disease recurrence after radiotherapy has been shown to be reduced by the use of neoadjuvant and adjuvant therapy in combination with radiotherapy.[15] Radiation causes injury to cells by inducing double-strand breaks in DNA through free-radical production.[16] Understanding the role of MTs in the resistance to radiotherapy may provide novel strategies for improving radiation sensitivity. We have previously shown that MT 2A, the predominant MT isoform expressed in the prostate, interacts with protein kinase D1 in vitro and in vivo.[17] The activity of protein kinase D1 can be altered by drugs such as bryostatin-1, which may influence the function of MTs. Alternatively, small molecular inhibitors can also be developed to influence the activity of MTs in PCa. Because the prostate gland in humans is readily accessible to imaging and therapeutic intervention such as direct needle injections, drugs altering MT activity may be administered directly into the prostate as a part of preparation for radiotherapy.

Our present study was limited to experiments performed using well-established PCa cell lines. Additional in vivo studies are needed to validate these findings. Although our data suggest a strong and significant association between MT induction and resistance to radiotherapy and cisplatin treatment, additional studies are needed to establish a direct causal relationship. Because resistance to cisplatin and radiotherapy can be due to a multitude of factors, altering the activity of MTs in PCa cells may not result in reversal of resistance to treatment in patients with PCa. Nevertheless, our study has established a useful model of MT induction by high concentrations of zinc similar to those in the human prostate gland and has provided strong evidence supporting the potential role of MTs in PCa cells.

CONCLUSIONS

We have established an excellent in vitro cell line model system to study MTs in PCa. Our results suggest that MT induction by zinc is associated with resistance to cisplatin and radiotherapy in PCa cells. Therapeutic targeting of MTs may therefore provide a means to overcome cisplatin and radiation resistance in PCa, although the varying concentrations of zinc within the heterogeneous population of prostate cells may pose additional therapeutic challenges.

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Appendix 2

Chapter 10

METALLOTHIONEINS AND PROSTATE CANCER

Narassa Narayani and Kethandapatti C. Balaji

Metallothionein (MT) is an avid metal-binding (metal + thiol/sulphur binding) protein in the human body. It binds to trace elements like zinc and copper as well as heavy metals like cadmium, and plays an important role in metal detoxification and homeostasis. MT isoforms are expressed differentially in benign and malignant prostate tissue, with increased MT expression noted in higher-Gleason-grade prostate cancer. MT expression in prostate has been shown to be regulated by high Zn concentration and promoter hypermethylation. MT is known to play a role in the resistance to chemotherapeutic agents such as cisplatin and radiation treatment, presumably by trace metal or free radical scavenging. MT expression in the prostate gland is of particular interest because heavy metals such as Zn, which is present at the highest concentration in prostate compared to other human organs, induce MT expression and may be amenable to therapeutic manipulation in order to improve sensitivity to chemotherapy and radiation. MT may prove to be a useful therapeutic target for novel approaches such as local or systemic heavy metal chelation therapy and gene vectors for treating patients with prostate cancer.

Keywords: Metallothioneins; prostate cancer; zinc; biomarker; treatment.

1. Introduction

Metallothioneins (MTs) are small-molecular-weight scavenger proteins that have been shown to play a role in normal development and in disease states such as cancer (Cherian *et al.*, 1993; Kägi and Schaffer, 1988). Among cancers in Western countries, prostate cancer is the most commonly diagnosed noncutaneous cancer in men and is associated with several thousand deaths each year (Hellerstedt and

Pienta, 2002; Jemal *et al.*, 2005). The role of MTs in prostate cancer is of particular interest because a variety of MT isoforms are differentially expressed in benign and malignant prostate tissue, and are associated with normal and disease development of the prostate gland (Theocharis *et al.*, 2004; Theocharis *et al.*, 2003). While the human prostate gland is not critical for survival in humans, it contributes to liquefaction of semen and plays an important role in fertility (Lwaleed *et al.*, 2004). In this chapter, we will discuss the expression of MT isoforms in prostate cancer, their association with prostate cancer, and the potential role of MT in disease development and in resistance to treatments such as radiation therapy and chemotherapy (Lazo *et al.*, 1998).

2. Prostate Cancer

2.1. *Prostate Gland has Highest Levels of Tissue Zinc Concentration Among Human Organs*

Prostate gland is unique among human organs because of its high levels of tissue Zn concentration compared to other organs (Costello and Franklin, 1998; Mawson and Fischer, 1951; Mawson and Fischer, 1952; Mawson and Fischer, 1953). Because MTs are metal-responsive proteins, the high concentration of Zn in prostate glands provides an important regulatory mechanism that is required for understanding MT expression in prostate gland and for its potential therapeutic manipulation to treat prostate diseases.

Interestingly, Zn concentration varies within prostate gland, with about 10-fold higher concentrations demonstrable in benign prostate compared to malignant prostate (Gyorkey *et al.*, 1967; Ogunlewe and Osegbe, 1989). Similar to Zn, citrate levels have also been demonstrated to decrease in prostate malignant tissue compared to benign glands (Costello and Franklin, 1998). Normally, mitochondrial aconitase, which is capable of oxidizing citrate, is kept inhibited by zinc in prostate gland; however, in prostate cancer cells, the low

concentration of zinc limits aconitase inhibition, leading to citric acid oxidation and reduction in tissue citrate levels. The low levels of citrate in malignant prostate have been exploited in magnetic resonance imaging (MRI) spectroscopy, where the tissue choline-to-citrate ratio detects prostate cancer within the gland with a positive predictive value of 90% and excludes the presence of cancer with an 83% negative predictive value (Kurhanewicz *et al.*, 2000; Westphalen *et al.*, 2007). Such an improvement in the clinical imaging of prostate provides the impetus to study additional molecular markers that may contribute to improve the clinical care of patients. MTs hold promise for patients with prostate cancer because their expression can be influenced by trace metal concentration (including Zn), and also because of their evolving role in prostate cancer development and in resistance to commonly used treatment modalities such as radiation therapy and chemotherapy.

2.2. *Expression of MT Isoforms in Prostate*

Depending on the age and the type of tissue, most adult mammalian tissue contains very low basal levels of MT. In the human body, it is synthesized primarily in the liver and kidneys. During early fetal development, MT is known to play the role of a temporary reservoir for essential metals like Zn and Cu. While in the early neonatal period, it is detected in the nucleus and the cytoplasm of the cell, in adult tissues it is mainly a cytoplasmic protein (Cherian *et al.*, 1994). A transient localization of MT into nucleus is seen during cell proliferation and differentiation under certain conditions like embryogenesis, early fetal development, and carcinogenesis. These changes in intracellular localization and expression of the MT gene are suggestive of MT being an oncodevelopmental tumor marker (Cherian *et al.*, 1994; Cherian *et al.*, 1993).

Since the discovery of MT in 1957 (Margoshes and Vallee, 1957), at least 10 isoforms of MT have been identified. They have been broadly subdivided into four major subgroups, MT-1 to

MT-4 (Moffatt and Denizeau, 1997). Of these isoforms, MT-1/MT-2, MT-2A, and more recently MT-3 (which was thought to be confined to neural tissue; see Uchida *et al.*, 1991) have been demonstrated in the human reproductive tissue. In the normal prostate gland itself, MT was seen to be localized more in the glandular epithelial cells of the peripheral zone than in the central zone of the prostate, indicating that this might be due to the functional difference among the prostatic epithelial cells (Suzuki *et al.*, 1991). Normal human prostate expresses the MT-1A, MT-1E, MT-1X, and MT-2A genes; however, MT-1X was not demonstrable in advanced prostate cancer (Garrett *et al.*, 2000).

Prostate cancer is commonly graded using the Gleason grading system, with an increasing grade correlating with high-risk disease and poorer clinical outcome (Albertsen *et al.*, 1998; Moussa *et al.*, 1997). MT expression has been shown to increase with a worsening Gleason grade, suggesting that MT expression may correlate with disease outcome. El Sharkawy *et al.* (2006) specifically addressed the MT-2 expression in human prostate tissue and identified high MT-2 expression in prostatic intraepithelial neoplasia (PIN), commonly considered to be a precursor of prostate cancer, and also noted that an increasing MT-2 expression correlated with a worsening Gleason grade. Contrary to prostate gland, seminal vesicles located adjacent to prostate gland rarely develop malignancy. While 70% of prostate tissue expressed MT, only 40% of seminal vesicle tissue stained for MT, suggesting that MT expression is correlated with an increased risk of malignancy (Pannek *et al.*, 2001).

2.3. *Role of MT in Prostate Cancer*

MT plays a main role in protecting the body against metal toxicity and from oxidative stress by acting as a free radical scavenger (Klaassen *et al.*, 1999; Thornalley and Vašák, 1985). MT has been demonstrated to have antioxidant, ~~and~~ anti-inflammatory, and antiapoptotic properties (Aschner and West, 2005; Penkowa *et al.*, 2006), and to play

an important role in cellular processes such as cellular proliferation and growth. MT also seems to be involved in regulation of the tumor suppressor protein p53 (Ostrakhovitch *et al.*, 2006). Malignant cells enriched with MT both *in vivo* and *in vitro* have shown to exhibit greater resistance to chemotherapeutic agents (Kelley *et al.*, 1988).

Studies have shown that MT is mainly a cytoplasmic protein in adult tissues, and is detected in the nucleus of normal cells in the early fetal and neonatal period (Chan and Cherian, 1993). When the level of intracellular MT expression increases, MT tends to localize to the nucleus, and by doing so causes rapid proliferation of the cells (Tohyama *et al.*, 1993), inhibits apoptosis (Kondo *et al.*, 1997), and moves the cells into the S phase (Tsujikawa *et al.*, 1991). Several studies have shown that increased MT expression may protect the cells from carcinogenic effects of cadmium or anticancer drugs (e.g. cisplatin, melphelan, chlorambucil) and from ionizing radiation (Bakka *et al.*, 1981; Kondo *et al.*, 1995b). However, this protection of the cells is not always seen when MT is overexpressed, but is seen to occur only when there is subcellular localization into the nucleus (Kondo *et al.*, 1995a).

MT also plays a major role in the detoxification of heavy metals. Cadmium, a heavy metal, is an environmental pollutant and is a major constituent of tobacco smoke. It has been classified as a toxic metal by the International Agency for Research on Cancer (IARC) as a known human carcinogen (Group 1; IARC, 1993). Exposure to this heavy metal, which has no known beneficial physiological role, has been linked to a wide range of detrimental effects on mammalian reproduction. Human studies indicate that nearly 7% of the general population suffers renal dysfunction from cadmium exposure (Klaassen *et al.*, 1999). Although it is not possible to quantify the contribution of cadmium to the incidence of prostate cancer (Waalkes and Rehm, 1994), cadmium and zinc have both been implicated as carcinogens for prostate cancer (Habib, 1980; Palmer, 1984; Waalkes and Rehm, 1994; Waalkes *et al.*, 1992). Cadmium is known to have androgen-like activity in the prostate, and has been implied to be the potential

mechanism for its carcinogenic effect (Ye *et al.*, 2000). It is known that African-American men in South Carolina have the highest age-adjusted death rate for prostate cancer in the United States (American Cancer Society, 2007); among the many factors is environmental exposure to cadmium and selenium through groundwater, as the soil in these rural areas has cadmium and selenium concentrations unique to South Carolina (Drake *et al.*, 2006). It is conceivable that there might be a possible interaction between the geological and underlying biological factors such as metal transporter gene expression by race in black men (Blackshear *et al.*, 2003).

In vitro and a few *in vivo* studies have shown that cadmium has the ability to stimulate the expression of the cellular proto-oncogenes c-*jun*, c-*fos*, and c-*myc* (Abshire *et al.*, 1996). Metallothionein null cells are more susceptible to chemical-induced apoptosis, while MT-I and MT-II knockout mice are sensitive to the cadmium-induced mRNA expression of c-jun and p53 (Zheng *et al.*, 1996). These data suggest that MT, a trace metal and heavy metal detoxifier, may be one of the important mechanisms that the body uses to prevent cadmium-induced prostate cancer.

2.4. *Regulation of MT Expression in Prostate Cancer*

Human MT synthesis is induced by metals such as Zn and Cd; by endogenous factors such as vitamin D, interferon, reactive oxygen species, interleukin 1, and glucocorticoids; and also by stress (Bremner, 1987). The level of the response to these inducers depends on the MT gene. The human MT gene is located on chromosome 16 (16q13) (West *et al.*, 1990). At least 10 of the 17 genes identified so far have been found to be functional. MT gene expression, which is metal- and isoform-specific, is controlled primarily at the level of transcription (Palmiter, 1998). MT genes present in their promoter-specific proximal sequences include metal response elements (MREs), glucocorticoid response elements (GREs), and

antioxidant response element. The transcriptional regulation of MT by metals depends on the metal-responsive transcription factor MTF-1 (metal transcription factor 1), a zinc finger transcription factor (Heuchel *et al.*, 1994) that is an important zinc sensor. MTF-1 in turn is under the control of a Zn-sensitive inhibitor termed MTI (metallothionein inhibitor) (Palmiter, 1994).

Differential expression of MT isoforms MT-1, MT-2, and MT-3 in response to Zn treatment has been demonstrated in human normal and malignant prostate cells and tissues (Wei *et al.*, 2008). The study provided evidence that there is attenuated MT-1/MT-2 expression with prostate tumor progression, and that the zinc induction of MT-1/MT-2 expression results in response to cellular zinc restoration. MT expression is upregulated under hypoxic conditions in prostate cancer cell lines and promotes cell survival (Yamasaki *et al.*, 2007).

MT expression is also regulated by promoter methylation and protein degradation. The MT-I promoter has been shown to be suppressed in human prostate cancer lines PC3 and DU145, probably by promoter methylation; whereas cadmium-induced MT-I in the human prostate cancer line LNCaP seems to be independent of promoter methylation. Degradation of MT protein is regulated primarily by the cellular Zn content and occurs in both lysosomal and nonlysosomal compartments (Chen and Failla, 1989), each being depleted and replenished at a different rate (Steinebach and Wolterbeek, 1992). Cytosolic MT is degraded by the cystosolic 26S proteosome complex (McKim *et al.*, 1992). The role of MT degradation specifically in prostate cancer remains to be investigated.

2.5. *Clinical Utility of MT in Prostate Cancer*

2.5.1. *MT as a Biomarker of Disease Progression in Prostate Cancer*

Studies using paraffinized human prostate tissue demonstrate both nuclear and cytoplasmic MT stains in benign and malignant prostate

tissue. An initial study by Zhang *et al.* (1996) demonstrated a positive correlation between increased MT staining and increasing Gleason grade in prostate cancer. Another study specifically studied MT-II isoform expression in hyperplastic, PIN, and neoplastic prostate human tissue. While epithelial cells in normal and benign prostatic tissues from 8 patients demonstrated patchy metallothionein staining, all 6 cases of PIN and 20 of 30 patients with prostatic carcinoma showed positive staining for metallothionein, which increased considerably from low-grade to high-grade tumors (El Sharkawy *et al.*, 2006). A study using imprint smears of prostate cancer tissue demonstrated a positive correlation between MT staining and nuclear proliferation, increasing Gleason score, pathologic stage, and disease recurrence (Athanassiadou *et al.*, 2007). While most studies demonstrate increased MT staining in prostate cancer, the MT-1G isoform has been reported to be downregulated in prostate cancer. Almost a quarter of 121 prostate cancer human samples had demonstrable hypermethylation of the MT-1G promoter, compared to none of the 13 benign prostate tissues examined.

Overall, the published studies suggest that expression of MT isoforms is altered in prostate cancer, and there is some preliminary evidence to suggest that MT may be useful as a biomarker of prostate cancer progression. However, larger studies specifically addressing the various MT isoforms and long-term clinical follow-up are necessary to establish the clinical utility of MT expression in prostate cancer.

2.5.2. *MT as a Target to Improve Sensitivity to Chemotherapy in Prostate Cancer*

Prostate cancer is generally considered resistant to chemotherapy (Kamradt *et al.*, 1999; Urakami *et al.*, 2005). Recently, two large randomized clinical trials demonstrated a modest improvement of 2 months ~~in patients receiving~~ docetaxol, a microtubule-stabilizing agent, in patients with advanced prostate cancer who failed first-line androgen ablative treatment (Petrylak *et al.*, 2004;

Tannock *et al.*, 2004). There continues to exist an urgent need to improve chemotherapy or sensitizing agents to current chemotherapy in order to improve the clinical outcome in patients with prostate cancer. Using prostate cancer cell line models, MTs have been shown to induce resistance to adriamycin in DU145 cells (Webber *et al.*, 1988). The ribozyme-induced loss of MT-2A mRNA induced cell death in prostate cancer PC-3 cell lines and was associated with dose-dependent downregulation of the proto-oncogene c-*myc* and the anti-apoptotic gene *Bcl*-2, suggesting that MT-2A is an important cell survival or antiapoptotic factor for prostate cancer cells (Tekur and Ho, 2002). The established role of MT in causing chemoresistance in other cancers, the upregulation of MT with advancing grade and stage of prostate cancer, and the inherent chemoresistance in prostate cancer strongly suggests a potential role for MT in prostate cancer as well. For example, cells with acquired resistance to cisplatin or chlorambucil overexpress ~~metallothionein~~, which tends to bind these alkylating agents to a higher extent than the nonresistant cells (Ebadi and Iversen, 1994). In addition to sequestering electrophilic anticancer drugs, ~~metallothionein~~ alters the therapeutic efficacy of antineoplastic agents by regulating the activities of zinc-requiring metalloenzymes or scavenging radical species (Ebadi and Iversen, 1994).

We have previously established an excellent *in vitro* cell line model to study the effect of MT expression in prostate cancer following treatment with Zn (Smith *et al.*, 2006). The results from our microarray analysis confirmed that zinc treatment induces 0.33% of the 12 144 genes studied, most of which are MTs, which were significantly associated with resistance to cisplatin in prostate cancer cells. Others have demonstrated the presence of ~~a metallothionein~~-like zinc-binding protein in prostate cancer cell lines exhibiting relative resistance to cisplatin, and nuclear localization of MT has been shown to be associated with cisplatin resistance in prostate cancer cell lines (Kondo *et al.*, 1995a; Metcalfe *et al.*, 1986). Although no specific MT inhibitor has yet been described, inhibition of cysteine

synthesis by propargylglycine has been shown to significantly reduce MT induction in mice inoculated with human or murine bladder tumor cells, which markedly enhanced the antitumor activity of cisplatin and other drugs (Saga *et al.*, 2004). Because MT may be therapeutically manipulated, further studies are needed to explore MT as a biomarker for the chemosensitivity of prostate cancer to cisplatin treatment and to possibly improve the efficacy of cisplatin as a radiosensitizer.

2.5.3. *MT as a Target to Improve Sensitivity to Radiation Treatment in Prostate Cancer*

Radiation to prostate is a primary treatment option for patients with prostate cancer, and has been shown to produce a satisfactory long-term clinical outcome (Altundag *et al.*, 2005; Bao *et al.*, 1991). However, about a third of patients undergoing radiation treatment eventually fail, emphasizing the need to improve long-term response to radiation treatment (Pollack *et al.*, 2003). MT plays a homeostatic role in the control and detoxification of heavy metals; several evidences indicate that MT has the capacity to scavenge reactive oxygen metabolite (ROM), particularly the hydroxyl radical. These substances — which are produced continuously during normal aerobic metabolism — may become noxious in situations of imbalance with endogenous antioxidants, leading to cellular destruction, chromosomal aberrations, and finally cancer. Paradoxically, by anticancer treatment such as radiotherapy and chemotherapy, tumor cells are killed by generating toxic amounts of ROM.

Prostate is a readily accessible organ that may be amenable to injection of local agents which could improve sensitivity to radiation. Cisplatin is a well-established chemotherapy drug that is known to improve sensitivity to radiation (Ebadi and Iversen, 1994). Alternatively, agents that influence MT expression either directly (such as gene vectors) or indirectly (through heavy metals such as Zn) can be

administered either directly into prostate or systemically to improve sensitivity to radiation treatment. EDTA, a heavy metal chelator, has been used in human clinical trials in patients with coronary artery disease with an established safety profile (Knudtson *et al.*, 2002). Using a general heavy metal chelator (EDTA) or a more Zn-specific chelator (TPEN) (Hashemi *et al.*, 2007) at the time of radiation treatment for prostate cancer (clinically not used yet) may decrease Zn-mediated MT induction and improve sensitivity to radiation. Such novel approaches could potentially improve the long-term clinical outcome to radiation treatment for prostate cancer and decrease disease recurrence. Clearly, further preclinical and clinical studies are needed prior to establishing MT as a useful target in patients undergoing radiation treatment for prostate cancer.

3. Conclusion

Metallothioneins (MTs) are trace metal and free radical scavenging small-molecular-weight proteins that are expressed in several human organs including the prostate, and seem to play an important role in development and cellular homeostasis. While the various isoforms of MT are expressed differentially in benign and malignant prostate tissue, MT expression increases with a worsening grade of prostate cancer. MT expression in prostate is of particular interest because heavy metals such as Zn, which is present at the highest concentration in prostate compared to other human organs, induce MT expression and may be amenable to therapeutic manipulation. In addition to induction by Zn, MT expression may also be regulated through promoter methylation in prostate tissue. MT has been demonstrated to play a role in the resistance to cisplatin chemotherapy and radiation treatment in prostate cancer. Because of the high concentration of Zn in prostate, MT may prove to be a useful therapeutic target in prostate cancer in order to improve the sensitivity to chemotherapy and radiation therapy treatments.

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